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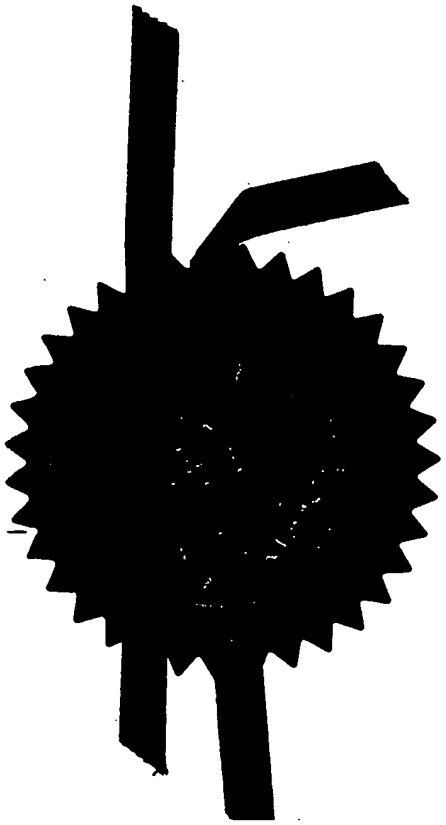

Signed W.J. Lowell

Dated 15 MAY 1995

PRIORITY DOCUMENT

For official use

THE PATENT OFFICE 31 MAY 1994 LONDON

31 MAY 1994
-2JUN 94#00400790 PAT 1 77 UC 25.00

Your reference IMA/FP5130604

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The Patent Office

Request for grant of a Patent

Form 1/77

Patents Act 1977

❶ Title of invention

1 Please give the title of the invention: MODIFICATION OF TETANUS TOXIN FOR USE AS A TRANSPORT PROTEIN

❷ Applicant's details

❑ **First or only applicant**

2a If you are applying as a corporate body please give:

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2b If you are applying as an individual or one of a partnership please give in full:

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Address: THE IMPERIAL COLLEGE OF SCIENCE, TECHNOLOGY AND MEDICINE
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Country: UNITED KINGDOM

ADP number *(if known)*: 04033452045

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Agent's address: YORK HOUSE
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5 Are you claiming that this application be treated as having been filed on the date of filing of an earlier application?

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8 Please supply duplicates of claim(s), abstract, description and drawing(s).

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Claim(s)		Description	26
Abstract	1	Drawing(s)	4

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MODIFICATION OF TETANUS TOXIN FOR USE AS A TRANSPORT PROTEIN

The present invention relates generally to the field of receptor-targeted biochemical delivery systems. More specifically, this invention relates to the use of modified polypeptide toxins as vehicles for delivering chemical compounds to cells bearing toxin receptors.

Tetanus toxin (TeTx) is a potent neurotoxin that induces paralysis by a mechanism involving the inhibition of neurotransmitter release. This *Clostridial* neurotoxin is initially produced as a single-chain protein of 150 kDa. Proteolytic cleavage then generates an active dichain molecule having a 100 kDa heavy (H) chain and a 50 kDa light (L) chain that are linked by a single interchain disulfide bond. Whereas the H chain mediates both the binding of the toxin to neuronal cell surface receptors and translocation of L chain into cells, the L chain is responsible for blocking the release of neurotransmitters. Although tetanus toxin causes persistent inhibition of synaptic vesicle exocytosis, it is not known to impair other aspects of neuronal cell physiology.

Another *Clostridial* neurotoxin is the botulinum toxin (BoNT). Recently, the neurotoxic mechanisms of tetanus and botulinum toxins have been elucidated. In particular, the TeTx-L chain was shown to possess a zinc-dependent protease activity having specificity for the vesicle-associated protein which is alternatively called synaptobrevin or vesicle-associated membrane protein (VAMP). The cleavage of such vesicle-associated proteins inhibits neurotransmitter release by preventing the fusion of transmitter-containing vesicles to the presynaptic membrane.

Whereas a single isoform of TeTx is produced by *Clostridium tetani*, seven serologically distinct isoforms of BoNT are produced by *Clostridium botulinum*. The seven botulinum toxin species are designated as BoNT/A-G. Both tetanus and botulinum type B neurotoxins are known to be zinc-dependent proteases. In *EMBO J.* **12**:4821 (1993), Blasi et al. proposed that the botulinum neurotoxin serotypes have evolved distinct substrate specificities while retaining a common protease activity. Whereas BoNT/A-G may cleave

different protein components of the exocytotic machinery, the consequenc is the same. Hence, it is believed that TeTx and the various botulinum tox. isoforms are both structurally and functionally related.

The various *Clostridial* neurotoxins use different cell surface receptors and cleave a variety of protein targets that are involved in vesicle function. Tetanus toxin, BoNT/B and BoNT/D all cleave VAMP-2, or Synaptobrevin-2. The BoNT/A and BoNT/E toxins cleave the membrane-associated SNAP-25 protein, while BoNT/C cleaves syntaxin. Tetanus, BoNT/D and BoNT/F have been reported to cleave Cellubrevin. The BoNT/F toxin cleaves VAMP, or Synaptobrevin. The target protein of the BoNT/G toxin is not yet identified.

As described above, all of the *Clostridial* neurotoxins bind to different cell surface receptors. Other neurons can also be affected, particularly at high toxin concentrations. The similar functional properties of the *Clostridial* toxins reflect common structural features.

Certain zinc-dependent endoproteases contain the conserved amino acid sequence HExxH. In thermolysin, zinc binding is achieved via His^{142} and His^{146} within this motif, together with Glu^{166}; the fourth ligand is water. Comparison of the sequence of BoNT and TeTx L chain with those of thermolysin and other zinc endoproteases has revealed the presence of the same consensus motif.

The role of Glu^{234} within this motif in the L chain of TeTx has been studied using site-directed mutagenesis and an assay for the proteolysis of cellubrevin. In *Nature* **364**:346 (1993), McMahon et al. demonstrated that the substrate cellubrevin was not cleaved when COS cells were cotransfected with mutant L chain (Glu^{234} substituted by Gln) and cellubrevin DNA constructs.

The present invention exploits the target specificity of the TeTx neurotoxin as a means for specifically delivering chemical agents to neuronal cells. Whereas the enzymatic properties of these toxins could be a disadvantage in such applications, the following invention overcomes this limitation.

One aspect of the present invention relates to a chemical conjugate for treating a nerve cell related disorder. This conjugate includes an active or inactive tetanus toxin having specificity for a target nerve cell. The inactive toxin is conjugated to a drug or other bioactive molecule without affecting the toxin's ability to enter the target nerve cell.

Further aspects of the present invention will be apparent to one having ordinary skill in the art upon reference to the ensuing detailed description.

Figure 1 is a schematic representation of the TeTx and the DNA construct used to express the MBP-L chain fusion proteins. In Figure 1A the single-letter code in the first part of the figure represents the amino acid sequence of the first several residues of the purified recombinant L chain and Ala^{234}-L chain determined by N-terminal microsequencing. Figure 1B illustrates the H chain disulfide bonded to the L chain. The location of the zinc-binding domain is also diagrammed.

Figure 2 is a graph showing the percentage of HV62 peptide cleaved by native, recombinant or mutant L chains as a function of time. The different symbols represent 33nM (open circles), 100nM (open squares) and 250 nM (filled triangles) of native L chain; or 250 nM recombinant L chain (open triangles); or 2.5 μM Ala^{234}-L chain (filled squares). The inset bar graph illustrates the ability of Ala^{234}-L chain to reduce the apparent hydrolysis of HV62 substrate by native L chain. The open bar represents the % substrate hydrolyzed by native L chain in the presence of Ala^{234}-L chain, while the hatched bar represents % substrate hydrolyzed in the absence of Ala^{234}-L chain.

Figure 3 is a graph representing muscle tension (as % of an initial value) as a function of time as an assay of neuromuscular transmission. The different symbols represent 10 nM TeTx (open circles), 10 nM reconstituted native H chain and L chain (open squares), 10 nM recombinant L chain assembled with native H chain (open triangles), 100 nM Ala^{234}-L chain refolded with H chain (filled squares). Values are the means ($\pm$SD) obtained from 3 experiments. The inset shows the results obtained with 20 nM reconstituted native H chain and L chain (open squares) and 40 nM reconstituted native H chain and

recombinant L chain (open triangles). Note that the stated concentrations reconstituted samples have not taken into account the minor content noncovalently linked chains.

Figure 4 is a schematic representation of the chemical synthetic scheme used to link the drug Vesamicol to a TeTx transporter using Maleimide.

The present invention relates to the use of a modified Tetanus neurotoxin as a transporter for the delivery of linked pharmacologic compounds. Among the compounds that will be linked to the toxin transporters are visualizable agents bearing fluorochromes, and drugs of therapeutic value. The contemplated cell populations that will be targeted by the toxin transporters include those which express cognate toxin receptors. Advantageous drug candidates are those that act intracellularly and can be rapidly introduced into the cell by the Tetanus toxin carrier as discussed in more detail below.

We discovered that an effective drug delivery agent may be prepared by the mutagenesis of a single amino acid position in the L chain of the Tetanus neurotoxin to inactivate its protease activity and then attaching a drug to that inactivated neurotoxin. Despite this abolition of enzymatic activity, the mutagenized toxin advantageously retained the ability to bind its cognate cell surface receptor. Of course, the scope of the present invention is intended to encompass any modified tetanus toxin molecule that is used as a transporter. For example, multiple insertions or deletions of amino acids in the zinc binding motif of tetanus toxin may attenuate the molecule in a similar fashion to the single amino acid modifications described herein. In addition, we have discovered other unexpected properties of the attenuated tetanus toxin molecule.

Significantly, we have discovered that both the heavy and L chains of the tetanus neurotoxin are required for optimal receptor-ligand interaction. In light of this finding, we reasoned that a toxin transporter would advantageously comprise both chains of the dichain molecule. Since the toxic properties associated with the L chain molecule could interfere with the therapeutic effect of a drug that was covalently linked to the toxin

transporter, we endeavored to create an attenuated L chain molecule that did not inhibit binding of the dichain molecule to the cognate toxin receptor. We discovered this could be accomplished, with apparently minimal disruption to the three-dimensional structure of the L chain protein, by mutation of a single amino acid position.

Accordingly, use of the inactivated tetanus toxin molecule as a vehicle that can be covalently linked to a drug has been explored. We discovered that reconstituted tetanus toxin, having an inactivated L chain disulfide-bonded to a native H chain, retained the ability to specifically interact with target receptors. Hence, the inactivated and chemically modified toxin complex can be used as a system for delivering linked chemical compounds to neuronal cells that express cell surface receptors for tetanus toxin.

Due to the fact that many individuals are immunized against tetanus toxin, it may be advantageous to further modify the tetanus toxin molecule so that it will be minimally neutralized by circulating antibodies. Modifications to the tetanus toxin molecule that retain its cellular binding and internalization ability, but limit its detection by the immune system are preferred.

As detailed below, an inactive TeTx L chain can be reassociated with the native H chain to form an inactive dichain toxin. This dichain molecule can serve as a receptor-targeted carrier for various chemical compounds. Due to the specificity of the H chain for its target receptor, a drug compound can be efficiently and specifically delivered to neuronal cells. Experiments wherein mice were injected with a reconstituted native tetanus toxin heavy chain and a non-attenuated recombinant light chain demostrated that this molecule was transported to the spinal chord and caused neuronal paralysis. This demonstrated that a recombinant, reconstituted tetanus toxin molecule acts in vivo as expected.

In the development of the present invention, the gene encoding TeTx-L chain was modified at the 5' end by the addition of a DNA sequence encoding a maltose-binding domain. This domain, therefore, was added to the N-terminal portion of the TeTx-L chain protein. Following expression in *E. coli*, the recombinant fusion protein (called MBP-L chain) was purified by affinity

chromatography. Proteolysis by factor Xa allowed separation of the T chain and the MBP domains. The purified L chain was then associated with purified H chain that had been isolated from *C. tetani*-derived TeTx to generate a dichain. This reconstituted TeTx molecule displayed activity characteristic of the native toxin.

In other experiments, modification of Glu^{234} to Ala in the TeTx-L chain abolished its ability to cleave VAMP or a synthetic substrate that contained the cleavage recognition site for TeTx-L chain. Advantageously, neurotoxicity of the complex formed by the mutant L chain and a wild-type H chain was also abolished, although the modified toxin retained the ability to bind its receptor.

By the methods described herein a mutant tetanus toxin can be synthesized. This toxin will retain its ability to bind neurons, even in the absence of an associated protease activity. This attenuated toxin can therefore facilitate the production of novel systems for the specific delivery of chemical agents to target neurons.

The mutagenized and enzymatically inactive dichain tetanus toxin described herein will advantageously serve as a neuropharmacologic transport agent for transporting chemical compounds to neuronal cells that express TeTx cell surface receptors. Such chemical compounds can be pharmacological agents, chemotherapeutic agents or visualizable agents that can be detected by light or other form of electromagnetic radiation.

Despite a number of similarities, those of ordinary skill in the art will appreciate that tetanus toxin is taken up by motor neurons and then transported to the spinal cord where it produces spasticity with convulsions. Thus, Tetx can reach target cells in the spinal cord by a pathway that begins in the muscles and traces back to the spinal cord.

Modified toxins based on TeTx are expected to carry linked drugs to the spinal cord along a neural pathway that connects the spinal cord and the injected muscle. Hence, a clinician using therapeutic agents based on the modified toxin-transporters of the following invention can selectively deliver drugs to the region of the spinal cord by injecting a TeTx-based therapeutic agent into an appropriate muscle.

The inactive tetanus toxin transporter will be primarily used to deliver drugs to target tissues for the purpose of controlling spasticity and excess movements in general areas, such as an arm, leg or portion of the body. The drug and transporter will be administered intramuscularly in one or more muscle groups which originate from the spinal cord target. In general, diseases affecting muscles below the neck are ideal targets.

Diseases that may benefit from such therapies include, but are not limited to, spasmodic torticollis, post stroke or traumatic brain injury induced spasticity, and dystonias of large muscle groups.

Table 1 outlines potential therapeutics related to the present invention. The entries in this table describe specific drug classes that can be linked to the tetanus toxin molecules. As indicated in Table 1, tetanus toxin will deliver therapeutic compounds to the spinal cord and other nerve cell sites.

Additionally, the use of both native and recombinant wild type tetanus neurotoxin proteins is intended to fall within the scope of the claims of our invention. In such applications, the enzymatic activity possessed by the L chain portion of the drug transporter will provide an added therapeutic advantage by virtue of its neurotoxic properties. For example, a drug that blocked nerve function could be linked to a wildtype tetanus toxin molecule to provide a compound that had a double action. The tetanus toxin molecule would provide its wildtype neuronal inhibitory effect, while the drug acted at its target site in the cell.

In addition, the clinical use of wildtype tetanus toxin alone is anticipated. A weak composition of tetanus toxin could be given to a patient to inhibit neuronal synapses in the spinal chord. Further, we intend our invention to be limited only by the use of tetanus neurotoxins as transporters, so that a wild-type toxin that is linked to a chemical compound, and which does not exhibit L chain proteolytic activity will also fall within the scope of the claims of our invention. Examples of neuromuscular maladies that will be investigated as therapeutic targets using active neurotoxins linked to drug molecules include: blepharospasm, strabismus, cerebral palsy and back pain due to muscle spasms.

As indicated below, some of the drugs selected act intracellularly other act extracellularly. As discussed herein, the intracellular drugs can be bound to a tetanus toxin carrier and efficiently internalized. However, drugs with extracellular actions can also be used in the present invention. We have discovered that reduced, alkylated tetanus toxin molecules can bind to the exterior of the cell, but will not be internalized. Thus, these reduced, alkylated molecules can be linked to extracellular-acting drugs and carried to the target cell surface. Once bound to the cell surface, enzymes such as esterases can cleave the drug from the tetanus carrier thus releasing the drug in close proximity to the target cell.

A brief description of the various uses of the transporter forms coupled with representative drug classes are summarized below.

Table 1

Therapeutic Uses of *Clostridial* Toxin Transporters

Transporter Molecule	Tissue Target	Drug Type	Mechanism of Action	Possible Clinical Outcome
Inactive-Intact-Tetanus Toxin	Spinal Cord	GABA agonist	Increase inhibitory neuron activity.	Block spasticity or cause a group of muscles to relax (at cord level), based on muscles injected.
Inactive-Intact-Tetanus Toxin	Spinal Cord	Neuronal Calcium Channel Blocker	Block nerve firing at spinal cord.	Block spasticity or cause a group of muscles to relax (at cord level), based on muscles injected.
Inactive-Intact-Tetanus Toxin	Spinal Cord	Adenosine agonist	Reduce firing of interneurons at the spinal cord.	Block spasticity or cause a group of muscles to relax (at cord level), based on muscles injected.
Inactive-Intact-Tetanus Toxin	Spinal Cord	Glutamate antagonist (or other EAA antag)	Reduce firing of interneurons at the spinal cord.	Block spasticity or cause a group of muscles to relax (at cord level), based on muscles injected.
Inactive-Intact-Tetanus Toxin or Active Toxin	Spinal Cord	Ricin or other protein synthesis toxins	Selective destruction of motor neurons in spinal cord.	Permanent paralysis.

The methods used to covalently couple the inactivated tetanus toxin and the chemical agents rely on conventional techniques that are familiar to those having ordinary skill in the art. The provision must be met however, that the domain of the compound that corresponds to the inactivated toxin retains the ability to specifically interact with cognate tetanus toxin receptor on target cells.

Modified tetanus toxin, produced according to the methods described above, will be stored in lyophilized form in containers under vacuum pressure.

Prior to lyophilization, the modified toxin will be combined with pharmaceutically acceptable excipients, including albumins and other appropriate agents as would be appreciated by those of ordinary skill in the art. Further information regarding such pharmaceutical preparations can be found in the "Physicians Desk Reference," published annually by Medical Economics Data of Oradell, New Jersey. The lyophilized material will be reconstituted with sterile non-preserved saline prior to intramuscular injection. This dissolved material will then be useful in the treatment of a variety of neuromuscular disorders as described above.

Methods of Linking Chemical Compounds to Light Chain Proteins

Whereas we contemplate that many different chemical compounds will be usefully bonded to toxin transporter molecules, a subset of these compounds will be neuropharmacologic agents or drugs. The following description therefore emphasizes methods of joining transporter proteins and drugs. However, those of ordinary skill in the art will appreciate the more generic term, "chemical compound" can reasonably be substituted for the term, "drug."

Many approaches are known for linking chemical compounds to the amino acid chains of proteins. We will use a linker molecule to separate the drug from the L chain peptide. As discussed above, we discovered that 11 amino acids can be attached to the N-terminus of the TeTx L chain with out substantially affecting its functionality. For this reason, we will use the N-terminal portion of the tetanus toxin L chains as the compound attachment point.

It is known that most drugs have positions that are not sensitive to steric hindrance. In addition, the linkage process should not introduce chirality into the drug molecule. Further, the linker and the drug should be attached through a covalent bond. The distance between the L chain and drug can be adjusted by the insertion of spacer moieties. Preferable spacers have functional groups capable of binding to the linker, drug and L chain and serving to conjugate them.

Preferred Spacers:

1) $HOOC-(CH_2)_n-COOH$, where n=1-12, suitable for insertion at the amino terminal end of a peptide, to connect it with a linker on a drug molecule.

2) $HO-(CH_2)_n-COOH$, where n>10, suitable for attachment at the amino terminal of a peptide to connect the L chain with a linker on a Drug molecule.

3) $(C_6H_6)_n$, where n>2, suitable for attachment to join the L chain with a linker on the Drug molecule. The benzene rings provide a rigid spacer between the Drug and L chain. Of course appropriate functional groups, for example as identified by X below, will be present on the benzene rings to link the drug and light chain.

Two different linker types are envisioned. In the first type, the Drug-Linker-L chain molecule remains intact after introduction into cells. In the second type, the Drug-Linker-L chain molecule is metabolized to free the drug after introduction into cells.

Linkers that remain intact after introduction

In one method, a cysteine residue is attached to the end of the L chain molecule by methods well known in the art. For instance, the gene construct that carries the L chain molecule can be mutated to include a cysteine residue at the N-terminal portion of the protein. A maleimide linker is then attached to the Cysteine residue by well known means.

In a second method, the linker is attached directly to the drug. A Drug-X moiety can have the following groups wherein X is OH, SH, NH_2, CONH, $CONH_2$. Of course, the proper group would not be in an active site or sterically hindered. The following reaction would link the Drug-X to the linker molecule.

Drug-X

$Br-CH_2-Linker$ ------> $Drug-X-CH_2-Linker$

Once the Drug has a linker attached, the following reaction can be used to link the Drug to the Toxin. In this reaction, the toxin has an accessible Lysine group that is used as the attachment point for the Drug. As discussed hereinabove, an extra amino acid, such as lysine, can be readily added to the N-terminal portion of the L chain gene and used as the attachment point for

a drug. In the following reaction, sodium cyanoborohydride is used to h the linker to the lysine group on the L chain molecule.

$$\text{Drug-Linker-CHO} \xrightarrow[\text{Toxin-Lys}]{\text{NaCNBH}_3} \text{Drug-Linker-CH}_2\text{-NH-Toxin}$$

Drugs that are envisioned to work in the present invention are those that have a free -XH group and can act as neuroinhibitors. These neuroinhibitors can interfere with the over-production of neurotransmitters in some medical indications such that the nerves will be inhibited from firing. Appropriate drugs with -XH groups are aconitine, adenosine agonists/antagonists, adrenergics, anatoxin A, antiepileptics, baclofen, brachiotoxin, brefeldin A, brevetoxin, captopril, curare, dantrolene, doxorubin, diazepam, grayanotoxin, lidocaine, methocarbamol, methyllycaconitine, neosaxitoxin, physostigmine, psychosine, THA, tetrodotoxin, vesamicol and vigabatum.

Linkers that cleave after introduction

Depending on the Drug's mode of action, it may be important for the Drug to be released from the L chain after introduction. In this method, the Drug has a free -XH group that is the active site for synthesis with a linker. The -XH group could be an alcohol, phenol, amine, carboxylic acid or thiol group.

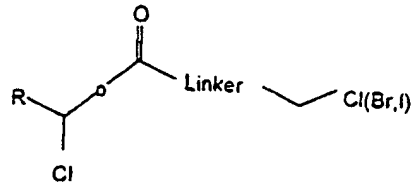
The general formula for linking a Drug to a toxin so that it will be metabolized after introduction is as follows:

$$\text{DRUG-XH + Linker} \xrightarrow{\text{Maleimide}} \text{DRUG-X-LINKER-Maleimide} \xrightarrow{\text{Toxin-SH}} \text{Drug-X-Linker-Maleimide-Toxin}$$

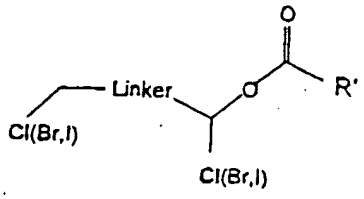
Where X can be OH, NH/NH_2, CO_2H, SH, $CONH_2$

Where the Linker can be A) or B) as detailed below:

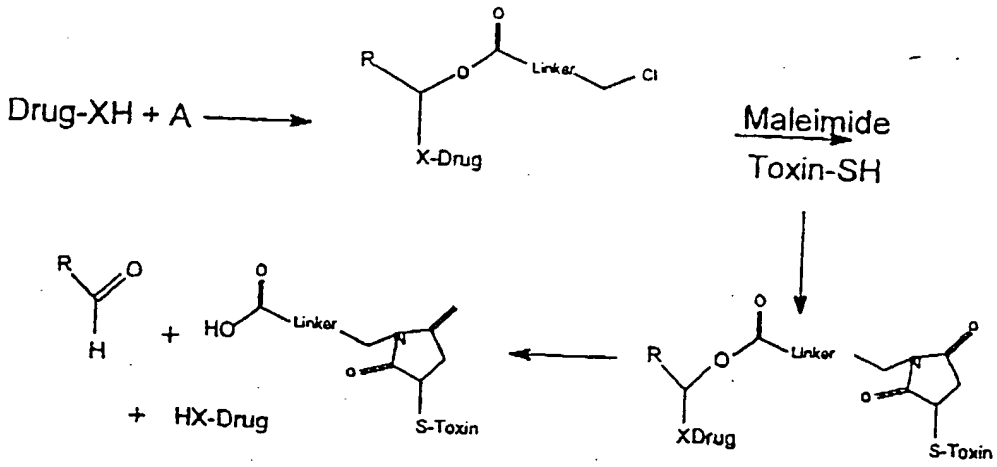
A)

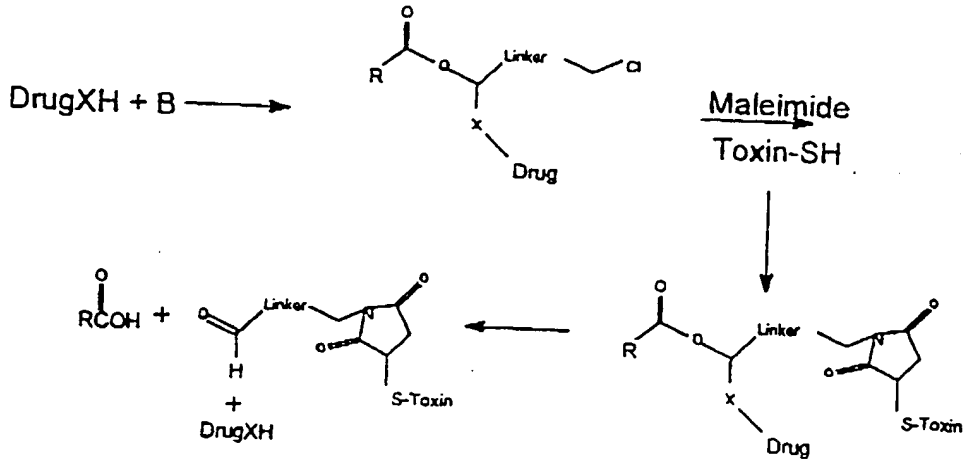


B)



The specific reactions with Linkers A or B are shown below.





Our strategy for linking ribozymes to the toxin transporters employs the free amine functional groups on adenosine and guanosine bases for linker attachment. In particular, our approach will be to incorporate modified adenosine or guanosine residues that are modified at their free amine positions with a linker that is in turn bound to the nitrogen position of succinimide. The structures of these modified nucleosides can be diagrammed as:

Sugar-Base-NH-Linker-Succinimide

Ribozymes are conventionally prepared by sequentially linking nucleosides in a defined order. The linking reaction occurs between the sugar moieties of the individual chemical units. Incorporation of a modified nucleoside, as described above, at either the 3' or 5' end of the ribozyme will provide a means for covalently linking to the toxin transporter according to the mechanism described previously.

Although other materials and methods similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. General references for methods that can be used to perform the various PCR and cloning procedures described herein can be found in *Molecular Cloning: A Laboratory Manual* (Sambrook et al. eds. Cold Spring Harbor Lab Publ. 1989) and *Current Protocols in Molecular Biology* (Ausubel et al. eds., Greene Publishing Associates and Wiley-Interscience 1987).

The initial step in creating an inactivated TeTx composition involved subcloning of the wild-type and mutated L chain structural genes into plasmid expression vectors. The vector employed for this purpose was designed to express a fusion protein that links a maltose binding protein domain at the N terminus, with L chain sequences at the C terminus. A vector-encoded factor Xa proteolytic cleavage site is interposed between the MBP and L chain insert sequences. Site-directed mutagenesis of the L chain DNA was employed to change Glu^{234} to Ala (Figure 1B).

Example 1 describes the methods used to create recombinant plasmids that encoded maltose-binding fusion proteins of wild-type and mutant tetanus toxin L chain.

Example 1

Preparation of Maltose-Binding-Protein-L Chain Constructs

E. coli K-12 strain TG1 was used as a host for the propagation of all plasmid constructs described below. Plasmid pMAL-LC (wild-type L chain gene) was constructed by polymerase chain reaction (PCR) amplification of a 1417-bp fragment encoding L chain from plasmid pTct87 that has been described by Fairweather et al., in *FEBS Lett.* **323**:218 (1993). The two polynucleotide primers, called a and d, that were employed in this PCR amplification had the sequences 5' GAGATGGTCGACATGCCAATAACCATAAATAAT-3' (SEQ ID NO: 1) and 5'-ACGCGAAGCTTTTATCATGCAGTTCTATTATA-3' (SEQ ID NO: 2), respectively. The amplification product of this reaction was digested with SalI and HindIII (Promega) and was then ligated to vector PMAL-c2 (Figure 1A) that had also been digested with the same enzymes. For site-directed mutagenesis, two additional primers, b and c, were used that had the sequences 5'-TAGTACATGTATAAGTGCGTGCATTAATAG-3' (SEQ ID NO: 3) and 5'-TTATACATGTACTACATGGT-3' (SEQ ID NO: 4), respectively. Each of these primers possessed AflIII cleavage sites that were used to mutate a Glu codon to an Ala codon at amino acid position 234 of the TeTx L chain. PCR amplification of Ptct87 was accomplished with primer pairs a/b and c/d, used separately. The amplification product from pair a/b was digested with SalI and AflIII, and that from pair c/d was digested with AflIII and HindIII. After purification with the MAGIC DNA CLEAN-UP SYSTEM (Promega), the samples were ligated to PMAL-c2 that had been cleaved with SalI and HindIII, creating PMAL L chain Aln^{234} (the mutated L chain gene).

After subcloning, colonies resistant to ampicillin were grown, the plasmid DNA was purified, and the construct (PMAL-LC) was confirmed using restriction enzymes and DNA sequencing of the whole insert. Thus, a SalI and HindIII digest yielded a fragment having the expected length of 1417 bp as determined by agarose gel electrophoresis. DNA sequencing confirmed that the nucleotide sequence at the junction of the 5'-end of the L chain gene and

the multiple cloning site (MCS) and the factor Xa cleavage site (Figure 1A) L chain sequence and the MBP coding sequences were all in the correct reading frame.

The availability of the plasmid constructs described above enabled the production of recombinant wild-type or mutant L chain fusion proteins. Specifically, cultures of bacterial clones that harbored plasmids PMAL-LC or PMAL-LC-Ala^{234} were induced with IPTG to stimulate high level synthesis of the recombinant fusion proteins. Large-scale purification of the two fusion proteins was accomplished by affinity chromatography of bacterial extracts on amylose affinity resin.

Example 2 describes the techniques used to produce and purify recombinant tetanus L chain fusion proteins encoded by the plasmid constructs described in Example 1.

Example 2

Expression of Fusion Proteins and Purification of Wild-Type and Ala^{234}-L Chain Mutant Proteins

E. coli clones harboring plasmids PMAL-LC or PMAL-LC-Ala^{234} were grown to densities of about 2 x 10^8 cells/Ml (A_{600nm} - 0.5) at 37°C in L-broth that was made 100 μg/Ml ampicillin and 2 mg/Ml glucose. Induction was initiated by the addition of isopropyl β-D-thiogalactoside (IPTG) to a final concentration of 0.3 mM. Cells were harvested 2 hours later and centrifuged at 6000 x g for 30 minutes. The resulting pellets were then resuspended in column buffer [10 mM Tris-Hcl, 200 mM NaCl, 1 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, and 1 mM dithiothreitol (DTT) (Ph 7.4)] containing 1 mM phenylmethanesulfonyl fluoride and lysed by sonication. After centrifugation, crude extracts were applied to an amylose affinity column (2.5 x 10 cm, 40 mL of resin). Following the removal of nonbound proteins by washing with buffer, the bound MBP-LC fusion proteins were eluted with column buffer containing 10 mM maltose according to the procedure described by Maina et al., in *Gene* **74**:365 (1988). The isolated fusion proteins were concentrated to 0.5-1 mg/mL using an Amicon CENTRICON. Protein samples were then analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting, using anti-MBP polyclonal

and anti-L chain monoclonal antibodies. SDS-PAGE of both cell extracts indicated the presence of an induced protein band (M_r - 90,000) that was absent from the Coomassie staining pattern of the noninduced cultures. The molecular weight of the protein band was in accordance with that expected from a fusion of MBP and L chain (M_r - 40,000 and 50,000, respectively). The optimal conditions established for expressing recombinant L chain and Ala^{234} mutant using the pMAL-c2 vector system were 2 hours of induction with IPTG at 37°C. Neither a longer induction time nor inclusion of protease inhibitors increased the yield of product. Both fusion proteins were soluble in aqueous buffer (up to 0.5 mg/mL) and stable for up to 8 months when stored at -20°C.

After this initial purification step, both MBP-L chain preparations were cleaved at 23°C for 24 hours with factor Xa at an enzyme:protein ratio of 0.5-1:100 (w/w). This cleavage gave complete conversion of the fusion proteins to the respective wild-type L chain and Ala^{234}-L chain with the liberation of MBP, as confirmed by SDS-PAGE. After extensive dialysis against the column buffer to remove maltose, L chain or Ala^{234}-L chain was further purified by reabsorption onto a new affinity column. The desired product from this purification step was found in the column wash fraction. Fractions of the column wash were monitored for A_{280nm} and checked again by SDS-PAGE and Western blotting.

For amino acid sequencing, recombinant L chain or its mutant was run on SDS-PAGE and transferred onto a poly(vinylidene difluoride) membrane as described by Tous et al., in *Anal. Biochem.* **179**:50 (1989), with automated Edman degradation performed on a Model 4000 protein sequencer (Chelsea Instruments, London). Microsequencing of the two products revealed four residues identical to those of the N-terminus of native L chain preceded by the 11 amino acids encoded by the multiple cloning site of the vector (Figure 1A). Given this success in producing recombinant L chain proteins having the desired structures, it remained to test the enzymatic activity of these compositions.

Measurement of the zinc-dependent protease activity of native L chain was employed as an assay for the activity of the recombinant L chain proteins. Two different protein substrates were used in this assay. In the first case,

bovine small synaptic vesicles (SSVs) were used. The assay for proteolytic cleavage of the substrate was based on Coomassie staining and Western blotting.

Example 3 describes the techniques used to assess the proteolytic activities of wild-type and mutant recombinant L chain proteins using SSVs as the substrate.

Example 3

Measurement of L Chain-Dependent Proteolysis of *in vitro* Substrates

Native, recombinant (wild-type or Ala^{234}) L chains were incubated with bovine small synaptic vesicles (SSVs) (0.5 mg/mL) for 90 minutes at 37°C in 50 mM Hepes, 400 mM NaCl, 5 mM DDT, 2 μM $ZnSO_4$ (pH 7.4). Reactions were terminated by the addition of SDS-PAGE sample buffer followed by boiling for 3-5 minutes. Samples were then subjected to SDS-PAGE and detected by Western blotting using affinity-purified anti-HV62 antibody raised against a 62-amino acid synthetic polypeptide corresponding to residues 33-94 of human VAMP 2 as defined by Shone et al., in *Eur. J. Biochem.* **217**:965 (1993). The method used to prepare the anti-HV62 antibody was essentially the same as the method described by de Paiva et al. in *J. Neurochem.* **61**:2338 (1993). Incubation of the recombinant (100 nM) or authentic (50 nM) L chain proteins with bovine SSVs resulted in proteolytic cleavage of VAMP, as semi-quantitatively assessed by Western blotting with the anti-VAMP antibody probe or protein staining of the digests following SDS-PAGE. The Ala^{234}-L chain proved inactive as a protease even at a concentration of 2.3 μM. This result highlighted the fact that Glu^{234} is essential for enzymatic activity.

To more accurately quantitate the relative activities of the native and recombinant L chains, RP-HPLC was used to measure the cleavage of a synthetic 62-residue polypeptide, HV62, corresponding to residues 33-94 of human VAMP-2.

Example 4 illustrates the procedure that was used to quantify the *in vitro* activities of native and recombinant L chains using the HV62 peptide substrate.

Example 4

Quantitation of the Proteolytic Activities of Native and Recombinant L Chain Proteins

A stock solution of HV62 peptide (40 μM final concentration, 60 μL final volume) in 20 mM Hepes and 200 mM NaCl (pH 7.4) containing 5 mM DTT was incubated at 37°C with L chain preparations (100 nM final concentration). At time intervals, the reactions were terminated by the addition of 60 μL of 5 mM ethylenediamine-tetraacetic acid and 1% (v/v) trifluoroacetic acid (TFA) (pH 2) followed by centrifugation. Samples were stored at -20°C until analysis. The amount of hydrolysis of HV62 was measured by reverse-phase high-pressure liquid chromatography (RP HPLC) on a Micropax C_{18} column equilibrated in 0.05% TFA using a 0-60% acetonitrile gradient, while monitoring at A_{220nm}. N-Terminal sequencing of the cleavage product confirmed a single proteolytic site between Gln^{76} and Phe^{77}, in accordance with the observations of Schiavo et al., as presented in *EMBO J.* **11**:3577 (1992). The percentage of HV62 hydrolysis was calculated from the peak height of the breakdown product that corresponded to residues 77-94. A linear standard curve that related peak height to known quantities of product was used for quantitation.

Quantitation of the separated cleavage product (residues 77-94), time- and concentration-dependent hydrolysis of the polypeptide by native L chain is presented in Figure 2. Cleavage of the HV62 substrate (40 μM) by recombinant L chain (250 nM) confirmed its proteolytic activity. However, a 2.5-fold higher concentration of the recombinant L chain required to exhibit the same level of hydrolysis ($n=4$) as that observed for the authentic L chain protein (Figure 2). Under the specified conditions, the initial rates ($n=4$) of substrate cleavage at 37°C with 100 nM native and recombinant L chain were 45.6 ± 3.6 and 21.6 ± 2.4 pmol/min, respectively. More importantly, proteolysis of the polypeptide (40 μM) was undetectable when Ala^{234}-L chain was incubated for 3 hours at 2.5 μM (Figure 2). This latter finding emphasized the importance of Glu^{234} to the catalytic activity of L chain.

The lack of proteolytic activity observed for the Ala^{234}-L chain mutant could either result from an inability to bind the substrate or to cleave the peptide bond (Gln Phe). To distinguish between these possibilities, the Ala^{234}

L chain was investigated for the ability to attenuate cleavage of the substrate by native L chain. This was simply tested by preincubating HV62 with Ala^{234} L chain before the addition of native L chain. To make this test, 9 μM HV62 was preincubated with 4.5 μM Ala^{234}-L chain in reaction buffer at 37•C for 1 hour before the addition of 150 nM native L chain. At the end of the reaction period, the sample was analyzed for substrate cleavage as described above. The results from this procedure indicated that presence of the Ala^{234}-L chain mutant protein reduced the activity of the native L chain by more than 50% (Figure 2, inset). This result indicated the mutant L chain retained the ability to bind peptide.

Given the demonstration that Ala^{234}-L chain possessed no detectable proteolytic activity, it was of interest to reassemble a dichain species from native H chain and inactive L chain components. This was of interest because only the H chain portion of the toxin possesses the ability to bind cell surface receptors. We reasoned that a dichain toxin which had lost the ability to proteolyze substrates could conceivably retain the ability to bind toxin receptors at the cell surface. Such a dichain species could be adapted for use as a vehicle for the delivery of various chemical species to neuronal cells.

Example 5 details the method used to prepare dichain TeTx that incorporates either native L chain, recombinant L chain or Ala^{234}-L chain.

Example 5

Reassociation of TeTx from Native H Chain and Recombinant L Chain

Equimolar amounts of either native, recombinant L chain or Ala^{234}-L chain were mixed separately with the same amount of H chain purified from TeTx, as detailed by Weller et al., in *Eur. J. Biochem.* **182**:649 (1989). The mixtures were dialyzed against 2 M urea, 20 mM DTT, 1 M NaCl, and 50 mM Tris-HCl (pH 8.4) with stirring for 18 hours and then further dialyzed without agitation against 50 mM Tris-HCl and 600 mM glycine (pH 8.4) for 72 hours. An aliquot (300 μg) was loaded onto an HPLC DEAE column in 25 mM Tris-HCl buffer (pH 8.4) and eluted with a NaCl gradient (0-1 M) in the same buffer. The extent of covalent reconstitution was checked by nonreducing SDS-PAGE and silver staining.

Results of this procedure indicated the reassociation of dichain species by virtue of the presence of stained protein bands that comigrated with native TeTx. With recombinant wild-type and mutated L chains the relative amounts of the dichain species were 55.1 and 56.8%, respectively, as determined by densitometric scanning of the silver-stained gel. Native H chain and L chain gave similar levels of reconstitution. The latter involved interchain disulfide formation as the toxin was converted back to free H chain and L chain upon reduction by DTT.

With the availability of reassociated dichain toxin molecules, it was of interest to test for the presence of biological activity. Although there was evidence that dichain species had reassociated, this alone was not evidence that the proteins were properly folded or that the appropriate inter- and intra-chain disulfide bonds had formed. Thus, it was necessary to perform a functional assay for toxin activity.

Example 6 describes the methods used to assess the biological activity of the reassociated dichain toxins.

Example 6

Bioassay of Reassociated Dichain Toxins

Mice (20 g) were injected (200 μL/mouse) subcutaneously into the dorsal neck region with dichain toxin or other samples as described by Fairweather et al., in *Infect. Immunol.* **58**:1323 (1990), and the LD_{50} values were determined, as described by Maisey et al., in *Eur. J. Biochem.* **177**:683 (1988). The results of this procedure are presented below in Table 2.

Table 2

Mouse Lethalities of TeTx and Reconstituted Samples Formed from Native HC and Recombinant LC or the Ala^{234} Mutant

sample	lethality in mice (LD_{50}/mg)[a]	covalent dimer[b] (%)
TeTx	0.5×10^8	100
reconstituted using		
native HC and LC	3.3×10^6	55.4
native HC and recombinant LC	3.3×10^6	55.1
native HC and Ala^{234}-LC	<50	56.8
native HC alone used for reconstitution	<50	

[a] Measured over 4 days, mean values are shown for triplicate experiments.
[b] HC purified from TeTx was reconstituted with equimolar amounts of native LC, recombinant LC, or Ala^{234}-LC to form dichains. The proportion of total protein present as a covalent dimer was determined by SDS-PAGE and densitometric scanning of silver-staining gels.

The results in Table 2 clearly indicate the dichain species reconstituted from the Ala^{234}-L chain and native H chain had no toxic activity beyond that of the H chain alone. This absence of activity was not due to the reassociation process because the dichain reconstituted from native H chain and the recombinant L chain did exhibit toxicity.

The documented local action of TeTx in blocking neuromuscular transmission that has been described by Habermann et al., in *Naunyn-Schmiedeberg's Arch. Pharmacol.* **311**:33 (1980) was also exploited to assess activity of the reconstituted samples relative to that of the intact toxin.

Example 7 describes the methods used to assess the ability of reconstituted dichain toxins to effect neuromuscular transmission.

Example 7

Effects of Reconstituted H Chain and Recombinant L Chain or Ala^{234}-L chain on Neuromuscular Transmission

The inhibition of acetylcholine release by the reconstituted dichain from mouse left phrenic nerve-hemidiaphragm preparations was measured as a reduction of the nerve-evoked muscle tension as described by de Palva

et al., in *FEBS Lett.* 277:171 (1990). The time to paralysis was recorded as the period from the addition of toxin to when the muscle tension decreased to 10% of the original amplitude as described by de Paiva et al. in *J. Neurochem.* **61**:2338 (1993). Results from this procedure are presented in Figure 3.

At 10 nM, TeTx abolished nerve-evoked muscle tension within 150 minutes, whereas toxin generated from native H chain and L chain required 240 minutes to achieve paralysis (Figure 3) this is in accord with the reported lower neuromuscular blocking activities of reconstituted chains from TeTx and BoNT/A, relative to those of their native toxins as determined by Weller et al., in *Eur. J. Biochem.* **182**:649 (1989), and Maisey et al., in *Eur. J. Biochem.* **177**:683 (1988). When recombinant TeTx L chain was reassembled with native H chain, the resultant dichain exhibited about one-half the expected potency; 40 nM recombinant dichain required the same paralysis time as 20 nM reconstituted native dichains (Figure 3, inset), consistent with the reduced enzymatic activity of expressed L chain noted above. With the mouse bioassay, TeTx also proved more toxic (13-fold) than the refolded native chains (Table 2), and again, when recombinant L chain was employed in the reconstitution, there was a further drop in lethality (Table 2), approximating that observed previously by Fairweather et al., as reported in *FEBS Lett.* **323**:218 (1993).

Example 8 describes how the native or recombinant tetanus toxin L chain proteins can be covalently linked to a chemical compound. In this Example, a drug that blocks uptake of acetylcholine from the cytoplasm to the synaptic vesicle is linked to the transporter protein using free SH groups. The synthetic pathway employed in this procedure is diagrammed in Figure 4.

Example 8

Chemical Bonding of Transporter Protein and Vesamicol

Vesamicol is first attached onto a linker of 1-chloropropyl 12-chloro-dodecanoate, using equimolar concentrations of each in a base catalyst solution (such as pyridine, 2,6-dimethylpyridine, triethylamine or tetramethylguanidine) in solvents such as THF, DMSO, DMF or acetonitrile. The reaction is performed at temperatures of between 0 and 100°C for 1 to 48

hours. The resulting vesamicol-linker product is then reacted with equimc amounts of the potassium salt of maleimide in the same solvents, as above, and in the presence of sodium iodide (used as a catalyst) using similar times and temperatures as above.

The recombinant inactive L chain and native H chain subunits are renatured to produce a dichain molecule of roughly 150 kDa M_r. Renaturation is accomplished by mixing equimolar amounts of L chain and H chain proteins in the presence of urea and dithiothreotol. The mixture is dialyzed at 4°C against a Tris-NaCl, (pH 8.0) buffer that contains, in mM, NaCl, 460; KCl, 10; $CaCl_2$, 11; $MgCl_2$, 25; $MgSO_4$, 28; Tris-HCl, 10. The buffer is preferably oxygenated during the renaturation process. The buffer is changed 5 times over 24 hours. The removal of urea and DTT leads to the disulfide linkage of the L chain and H chain. Each dichain has several free sulfhydryl groups that are available for drug attachment.

The vesamicol linker is bonded to the free sulfhydryl groups found on the intact transporter molecule by mixing a 5 fold molar excess of the vesamicol linker with the transporter in Tris-NaCl, described above, at 4°C in the dark for 1 to 24 hours. The transporter-vesamicol preparation is then dialyzed against Tris-NaCl overnight to remove excess vesamicol-linker-maleimide from the vesamicol transporter.

The drug-transporter material is then available for administration as a sterile injection in a therapeutically effective dose.

Convincingly, the dichain toxin reconstituted using Ala^{234}-L chain and native H chain proved inactive on neuromuscular transmission over 6 hours at 100 nM (Figure 3). These findings confirmed an essential role for the enzymatic activity in the toxin's action.

The modified and inactivated TeTx neurotoxin transporter described above will have numerous clinical applications. For example, we anticipate these modified toxins will be useful in the treatment of neuromuscular disorders that affect spasticity in general areas of the body. These disorders include, but are not limited to, spasmodic torticollis, post-stroke or traumatic brain injury induced spasticity.

Example 9 describes how the chemically modified, inactive TeTx transporter described above can be used as a therapeutic agent for delivering chemical compounds to neurons that express toxin receptors.

Example 9

Therapeutic Administration of Modified Toxins:
Spasmodic Torticollis (Cervical Dystonia)

A female, age 45, suffering from spasmodic Torticollis, as manifested by spasmodic or tonic contractions of the neck musculature, producing stereotyped abnormal deviations of the heard, the chin, being rotated to one side, and the shoulder being elevated toward the side at which the head is rotated, is treated by therapeutically effective doses of an appropriate drug such as those discussed in Table 1, as would be appreciated by one of ordinary skill in the art, attached to an inactive tetanus toxin transporter directly into the affected muscles. After 3-7 days, the symptoms are substantially alleviated, i.e., the patient is able to hold her head and shoulder in a normal position or there is a dramatic reduction in the pain and discomfort.

Example 10 further illustrates how the chemically modified, inactive TeTx transporter described above can be used as a therapeutic agent for delivering chemical compounds to neurons that express toxin receptors.

Example 10

Therapeutic Administration of Modified Tetanus Toxin:
Post Stroke or Traumatic Brain Injury Induced Spasticity

A young male, age 24, suffering from traumatic brain injury, has developed upper and lower limb spasticity which restricts movement and impedes rehabilitation and hygiene. Symptoms include severe closing of the hand and curling of the wrist and closing of the legs such that the patient and attendant have difficulty with hygiene. In addition, the spastic nature of the limb impedes physical rehabilitation and causes muscle contracture and possibly joint immobilization. Sterile injections of therapeutically effective doses of an appropriate drug, such as those disclosed in Table 1 as would be appreciated by one of ordinary skill in the art, attached to an inactive tetanus toxin transporter are administered directly into the affected muscles.

A female, age 70, suffering from a cerebral vascular event (stroke, developed lower limb spasticities which require extensive efforts to maintain hygiene. The patient is injected in both limbs with therapeutically effective doses of an appropriate drug, as would be appreciated by one of ordinary skill in the art, attached to an inactive tetanus toxin transporter. Injections are made directly into the affected muscles. Relief of these symptoms occur in 7-21 days such that the lower limbs have relaxed enough to allow the patient and attendant to perform normal hygiene.

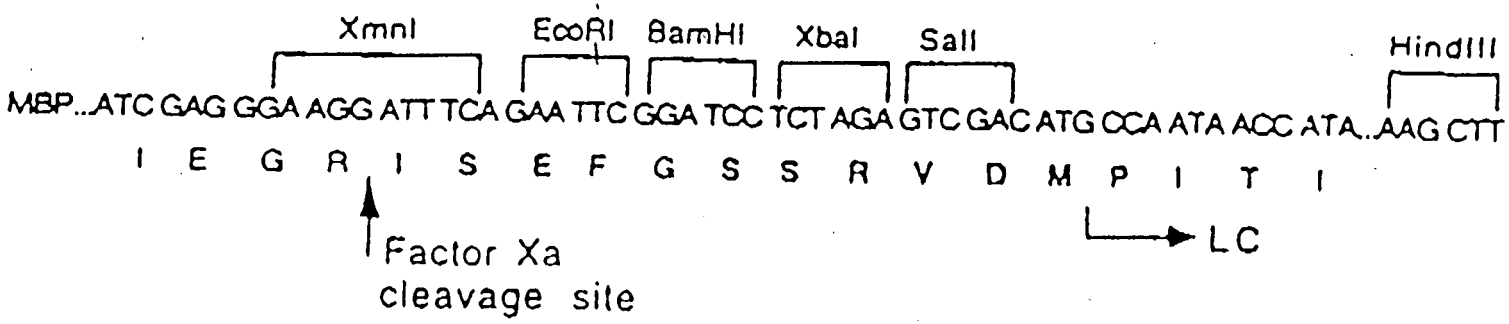
In summary, we have gained further insight into the action of tetanus toxin by employing recombinant DNA techniques to produce a L chain protein in useful quantities. Utilizing a PCR-based protocol, the gene encoding the L chain was amplified, subsequently cloned into expression vectors and expressed at a high level in *E. coli*. Moreover, once enzymatically cleaved from the maltose binding protein, the recombinant L chain protein showed properties like those of the native proteins. Also, the expressed tetanus L chain was reconstituted with purified native H chain to form disulphide linked, dichain proteins which inhibited nerve-evoked neuromuscular transmission *in vitro*.

Most significantly, we also discovered that single amino acid substitutions in the sequence of the L chain proteins completely abrogated the proteolytic activity ordinarily associated with the wild-type proteins. This now allows the formation of dichain toxins that are attenuated by virtue of incorporating a proteolytically inactive L chain subunit.

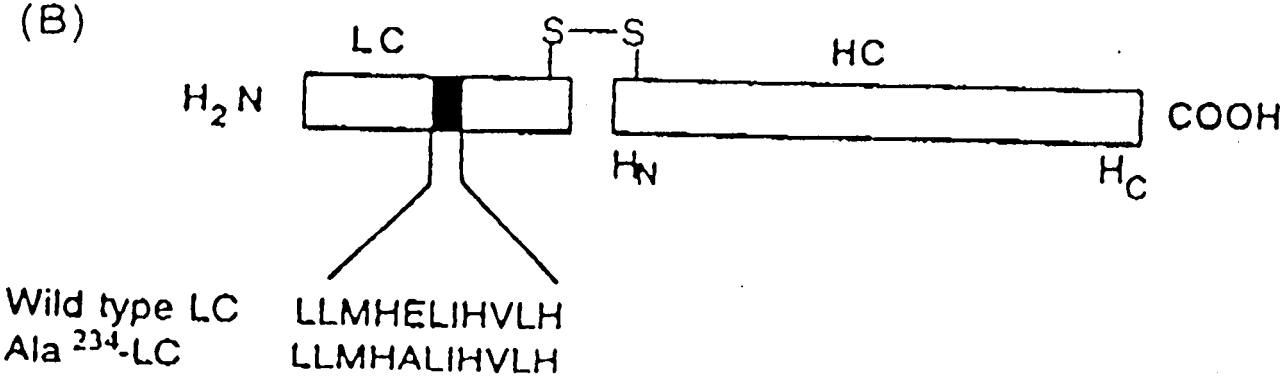
Abstract of the Invention

A chemical conjugate for treating a nerve cell related disorder is provided. This conjugate includes an active or inactive tetanus toxin having specificity for a target nerve cell. The toxin is conjugated to a drug or other bioactive molecule without affecting the toxin's ability to enter the target nerve cell.

(A)


XmnI
EcoRI
BamHI
XbaI
SalI
HindIII
MBP...ATC GAG GGA AGG ATT TCA GAA TTC GGA TCC TCT AGA GTC GAC ATG CCA ATA ACC ATA...AAG CTT
I E G R I S E F G S S R V D M P I T I
Factor Xa cleavage site
LC

(B)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S—S
HC
H2N
COOH
HN
HC
Wild type LC LLMHELIHVLH
Ala 234-LC LLMHALIHVLH

fig. 1

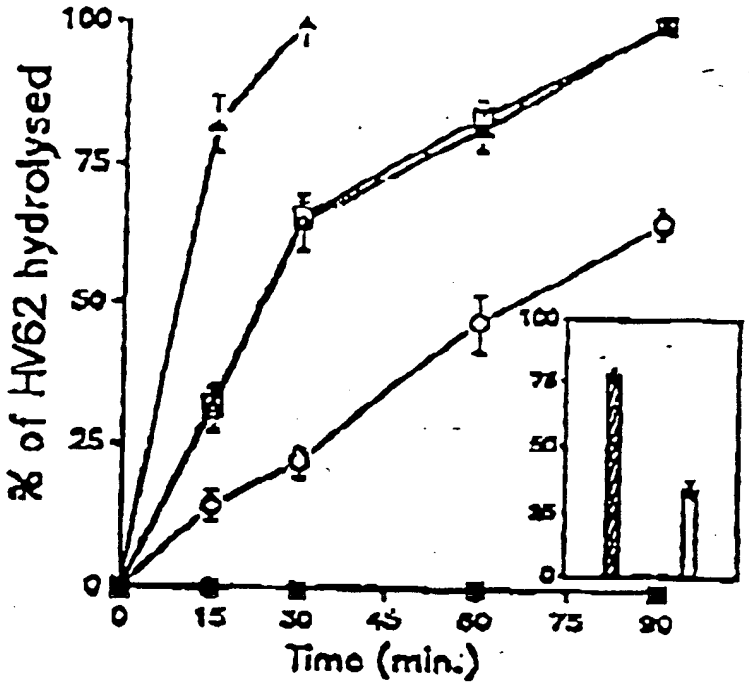

Fig. 2

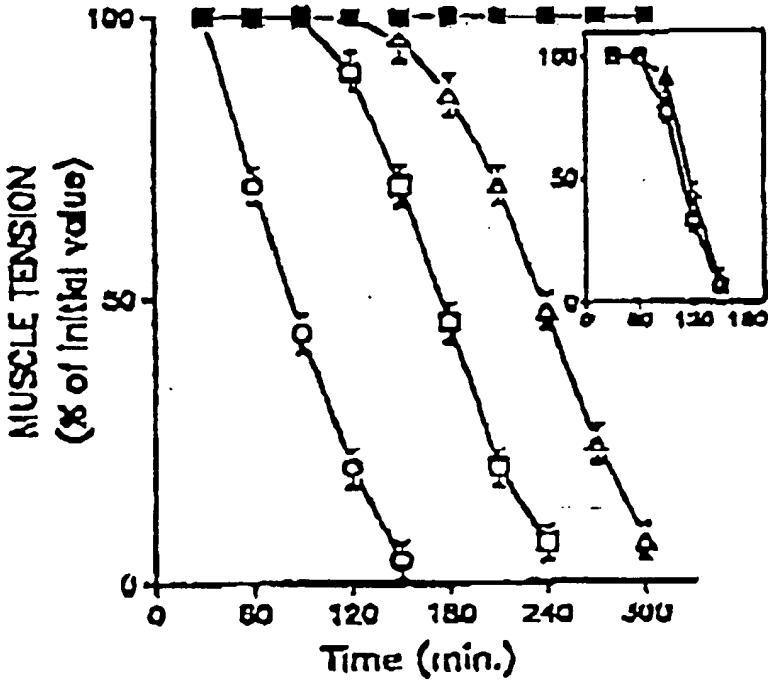
MUSCLE TENSION
(% of initial value)
100
50
0
0
60
120
180
240
300
Time (min.)

Fig. 3

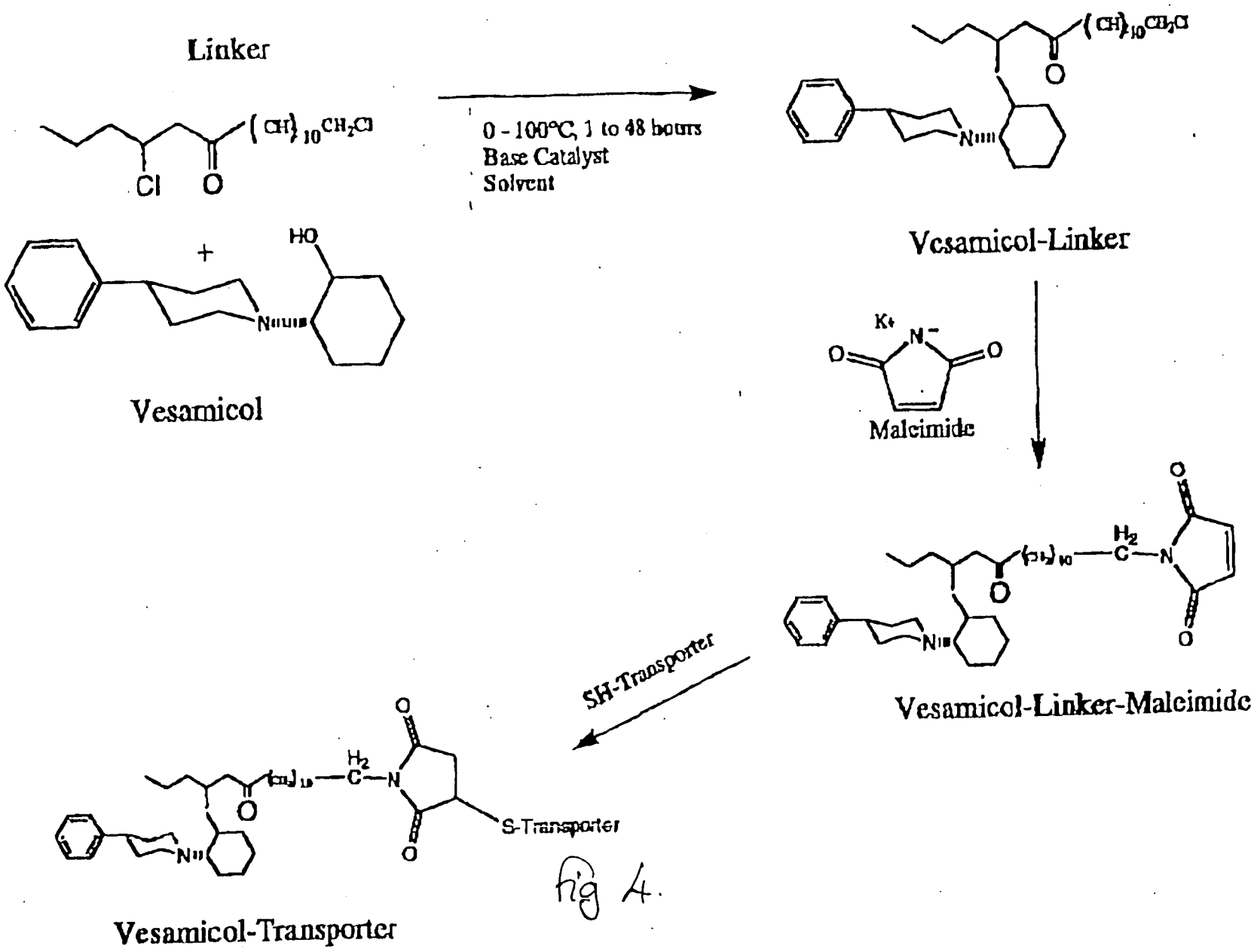
Linker
0 - 100°C, 1 to 48 hours
Base Catalyst
Solvent
Vesamicol
Vesamicol-Linker
Maleimide
Vesamicol-Linker-Maleimide
SH-Transporter
S-Transporter
Vesamicol-Transporter

fig 4.